

INVOLVEMENT OF MICROBODIES IN  
SYMBIOTIC NITROGEN-FIXING ARACHIS  
HYPOGAEA L. (PEANUT) ROOT NODULES

CENTRE FOR NEWFOUNDLAND STUDIES

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SOHAIL HAMEED









INVOLVEMENT OF MICROBODIES IN SYMBIOTIC  
NITROGEN-FIXING ARACHIS HYPOGAEA L. (PEANUT) ROOT NODULES.

BY

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Studies in partial fulfilment of the  
requirements for the degree of  
Master of Science

Department of Biology  
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## ABSTRACT

Studies were undertaken to examine the ultrastructure and enzyme activities of peanut, Arachis hypogaea L. Var. Jumbo virginia root nodules induced by the nitrogen-fixing rhizobial strain, Bradyrhizobium sp. 32H1. Root nodules at different stages of development were assayed for their nitrogen-fixing ability using the acetylene reduction technique. Highest values for nitrogen fixation were obtained in 35 days old nodules. The bacteroids and the host cytosol/organelle fractions of root nodules were assayed for catalase activity. Catalase activity was present in all fractions, being highest in the bacteroids. The in vitro grown 32H1 were also assayed for catalase activity. There was negligible or no catalase activity in intact bacteria, but the supernatant from-broken bacteria showed considerable catalase activity.

Peanut root nodule anatomy differs considerably from the nodules of other nitrogen-fixing legumes. The central infected zone is devoid of any uninfected cells except for the rays of cortical cells running through the infected cells, which divide them into several masses. Sections fixed in a mixture of paraformaldehyde and glutaraldehyde were processed for ultrastructural studies. The 3,3'-diaminobenzidine (DAB) reaction for localization of catalase was used. Controls were run by adding 3-amino-1,2,4-triazole (AT), potassium cyanide (KCN) in the incubation mixture and also by pre-boiling sections or incubating the sections without DAB and hydrogen peroxide.

The characteristic ultrastructural arrangement and differentiation of rhizobia into large spherical bacteroids was observed within the host cells.

Besides other organelles, lipid bodies were present in abundance and in close association with the bacteroids. Often they were seen attached to the peribacteroidal membrane envelope. Microbodies were observed in both uninfected and infected cells at all stages of nodule development unlike other nitrogen-fixing legumes where they are only found in uninfected cells. The microbodies in peanut root nodules were in close association and often in contact with the peribacteroidal membrane envelope and the bacteroids. Occasionally their membranes were seen fusing with the peribacteroidal membrane envelope. Microbodies were found to be DAB positive and the osmiophilic electron dense reaction product was found within its matrix. The presence of abundant lipid bodies, their physical contact with the peribacteroidal membrane envelope and with the bacteroids along with the microbodies and high catalase activity suggest that the lipids may be utilized as a carbon source during symbiosis through  $\beta$ -oxidation pathway.

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Besides the lipid bodies and the microbodies another osmiophilic DAB positive structure called the dense body was observed associated with the bacteroids. It resembled the central core of the microbody, lacked a membrane and was present at the interface between the peribacteroidal membrane envelope and the outer bacteroidal membrane and also within the matrix of the bacteroids. The DAB positive reaction shown by the dense bodies and their association with the bacteroids indicate that there is a possible role for them in the breakdown of hydrogen peroxide, produced during lipid utilization.

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## Chapter 1

# INTRODUCTION

### 1.1. General Introduction.

Nitrogen fixation is the process of combining free atmospheric nitrogen ( $N_2$ ) with other elements such as hydrogen and oxygen making it readily available to living organisms. Fixed nitrogen is of prime importance to all living forms, for it is an essential component of nucleic acids and proteins. It is one of the major nutrients of food and acts as a limiting factor in the production of balanced diets.

Nitrogen is a major constituent of the biosphere. The atmospheric dinitrogen occupies approximately 75% by weight and 78% by volume of the atmosphere and totals  $3.9 \times 10^{15}$  metric tons. The bulk of the nitrogen in the lithosphere is in the fundamental rocks, in the form of dinitrogen and amounts to 50 times that present in the atmosphere (Burns and Hardy, 1975). Dinitrogen is chemically very unreactive and unavailable to the higher (eukaryotes) organisms.

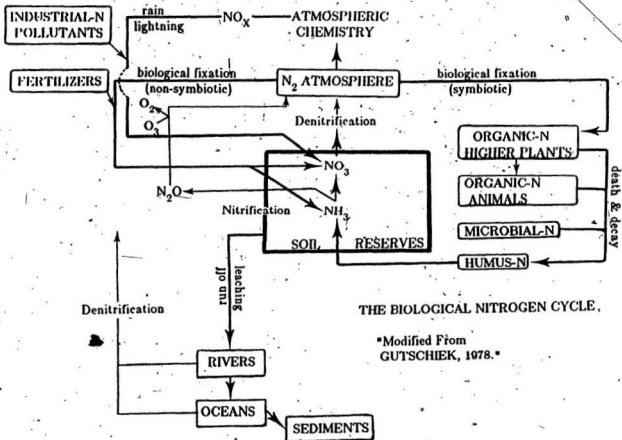
Nitrogen utilization by an organism involves its conversion to more reactive forms, usually to the highly oxidized states as nitrates, nitrites or to the reduced state as ammonia. Such conversions occur through physical processes such as lightning, U.V. irradiation and internal combustion engines or through biological

processes such as symbiotic and asymbiotic nitrogen fixation. However, these are not unidirectional processes and nitrogen is cycled through a series of interrelated processes adding it back to the nitrogen reservoirs (Fig. 1-1).

The biological fixation of nitrogen, symbiotic or asymbiotic, which is carried out by the enzymatic activity of certain prokaryotic microorganisms, is the major source of renewable combined nitrogen available to the biosphere (Postgate, 1982a). The biologically fixed nitrogen amounts to about  $122 \times 10^6$  metric tons per annum (Burris, 1980). This accounts for almost 70% of the world's fixed nitrogen in soil and water. Fertilizers contribute approximately 15% and environmental factors contribute the other 10% of the fixed nitrogen (Bray, 1983).

Industrial nitrogen fixation (N-fertilizers) contributes a significant amount of the fixed nitrogen to the biosphere. Its annual production for the years 1982-1983 was  $63.40 \times 10^6$  metric tonnes and its annual consumption for the same period was  $61 \times 10^6$  metric tonnes (FAO, 1983). However, this amount is far less than the amount fixed by biological processes. Industrial fixation is a high energy-demanding process and is becoming an increasingly expensive source of fixed nitrogen due to high energy costs. However, the biological process is also highly energy dependent, but when coupled with photosynthesis, can limit the use of any other energy source. Due to the high cost of fertilizers, research emphasis is being given to the biological fixation of nitrogen to compensate for the net loss of nitrogen to the atmosphere through denitrification. The biological nitrogen fixation process can also be used to increase the crop productivity in agricultural practices to fulfil the energy requirements in the forthcoming years.

**Figure 1-1:** A simplified diagram of the biological nitrogen cycle showing the pathway through which nitrogen is cycled between the atmosphere and the biosphere. Modified from Gutschick (1978).



The biological fixation of nitrogen is carried out by blue-green algae, actinomycetes and bacteria such as, Clostridium, Azotobacter and Rhizobium, respectively. There are two major categories of nitrogen-fixing systems according to the types of organisms involved, i.e., Nitrogen fixation by free living microorganisms and Nitrogen fixation by symbiotic association, which is either associative symbiosis or root-nodule-forming symbiosis (Stewart, 1977).

Non-symbiotic nitrogen fixation. In free-living systems the prokaryotic microorganisms fix nitrogen without a host. Bacteria are dominant among the soil nitrogen-fixing organisms, whereas blue-green algae predominate in the aquatic environment. Nitrogen fixed by free-living microorganisms is not directly available to the higher plants for it is taken up only after the death and decomposition of the microbe. It has been estimated that free-living nitrogen-fixers are about 1,000 times less effective in their contribution of fixed nitrogen to the soil than is a good symbiotic association (Bray, 1983).

Symbiotic nitrogen fixation. Symbiotic nitrogen fixation is either associative symbiosis, between two microorganisms, e.g. between Paspalum notatum and Azotobacter paspali or between Digitaria decumbens and Spirillum lipoferum, or it is nodule-forming symbiosis, e.g. Rhizobium - legume association, Rhizobium - non-legume association, Actinomycete - non-legume association and Cycad - blue-green algae association. From the agricultural point of view, Rhizobium-legume symbiosis is recognized as one of the most important systems, because it is a major source of fixed nitrogen available to the crop plants.

## 1.2. Rhizobium-Legume Symbiosis.

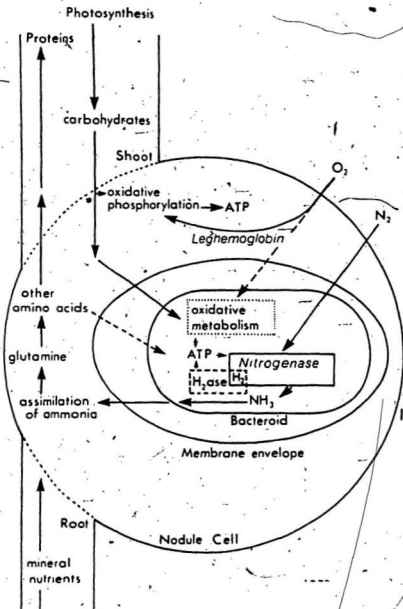
The legumes belong to *Fabaceae* or *Leguminosae*, the third largest family of flowering plants in the world. It has three major sub-families: *Papilionoideae*, *Mimosoideae* and *Caesalpinioideae*. The family *Fabaceae* contains about 750 genera and 18,000 - 19,000 species (Allen and Allen, 1981). It has a worldwide distribution and is found in temperate, tropical and arctic climates. It includes plants like peas, beans, alfalfa, clover, lupin, soybean, cowpea and peanuts. The family is characterized by its fruit, the legume. The majority of the members of this family show a characteristic symbiotic association with the nitrogen-fixing bacterium Rhizobium. Of all the species examined for nodulation, 85% of the species of the sub-family *Papilionoideae* produce effective nodules and fix nitrogen. Symbiotic nitrogen fixation is found in about 25% of the members of the sub-family *Mimosoideae* and is rare among the subfamily members of *Caesalpinioideae* (Postgate, 1982b). The nodules are generally restricted to the roots, however, the tropical marsh legume, Sesbiana rostrata develops nodules on the stem (Dreyfus and Dommergues, 1981).

The nodules induced by Rhizobium are specialized structures in which some of the host cells are inhabited by the symbiotic rhizobial partner. In general, the prokaryotic partner contains the enzyme nitrogenase, which reduces dinitrogen to ammonia. The eukaryotic partner (the host plant) assimilates the ammonia into its own organic molecules and provides the microorganism with a suitable environment in which to live (Fig. 1-2).

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**Figure 1-2:** A schematic representation showing the interaction between the microorganism (bacteroid) and the host plant (legume) and the biological components involved in symbiotic nitrogen fixation. (Verma, 1980).





Rhizobium is a genus of gram-negative, nitrogen-fixing bacteria belonging to the family Rhizobiaceae which usually produces root nodules in legumes (Beringer *et al.*, 1979). They are usually rod-shaped with a typical cell wall envelope of a gram negative bacteria. They are approximately  $0.5-0.9\mu\text{m} \times 1.2-3.0\mu\text{m}$  and can occur singly or in multiple numbers forming rosettes. They are commonly pleomorphic under adverse growth conditions. The intercellular inclusions include a central nucleoid (DNA) in a homogeneous cytoplasmic matrix interspersed with a large number of poly- $\beta$ -hydroxybutyrate granules, glycogen and ribosomes. They are generally motile, having one polar or sub-polar flagellum, or two to six peritrichous flagella. The genus Rhizobium is aerobic, possessing oxidative respiratory metabolism with oxygen as the terminal electron acceptor and grows well under low oxygen concentration. They grow best under a temperature range of  $25^{\circ} - 30^{\circ}\text{C}$  and a pH range of 6.0 - 7.0. Their colonies on agar medium are about 2 - 4mm in diameter and appear circular, convex, semitranslucent and mucilaginous. On the basis of differential growth rates they are divided into two groups, fast growers and slow growers (Quispel, 1974). According to the new classification in Bergey's Manual (Jordan, 1984), the fast growers are classified as Rhizobium and the slow growers as Bradyrhizobium. The fast growers have a mean generation time of 2 - 4 hours and the slow growers have a mean generation time of 6 - 8 hours (Vincent, 1977).

The important feature of free-living Rhizobium is its ability to infect specific legume hosts, differentiate into bacteroids within the host root nodule and fix atmospheric nitrogen symbiotically. The bacteroid state can also be induced in

cultures (Jordan, 1982). The bacteroids are characterized by highly irregular shape resulting from changes in the outer cell wall envelope (Van Brussel, 1973; Sutton and Paterson, 1979; Bal *et al.*, 1980). Rhizobia in general produce various indole compounds (Vincent, 1977). Tetracycline antibiotics are generally active against rhizobia, although they are susceptible to a wide spectrum of antibiotics. Antagonism is exhibited towards rhizobia by fungi, actinomycetes, eubacteria and by rhizobia itself. Members of the genera Agrobacterium, also belonging to the family Rhizobiaceae, show close relationship with Rhizobium. All species of Agrobacterium, with the exception of A. radiobacter incite cortical hypertrophies on plants. Other soil microorganisms such as protozoans, myxobacteria and Bdellovibrio are the predators of rhizobia. Lysogeny caused by rhizophages has been recorded (Vincent, 1977). Data related to agglutination, precipitation reactions, complement fixation and antibody absorption have helped a great deal in the understanding the chemical structure, taxonomy and identity of rhizobia (Vincent, 1977).

Before penetrating the root of the plant, Rhizobium is found highly concentrated in the rhizosphere region (Quispel, 1974; Brock, 1979). This is due to the release of metabolites by the plant which attract the Rhizobium and support their heterotrophic growth in the soil (Sharifi, 1984). The rhizosphere also harbours various other forms of microbes such as protozoa, algae, fungi, nematodes and bacteria. The thickness of the rhizosphere varies from species to species depending upon the nature of root exudates and the water content. The plant exudates, which include sugars, amino acids and vitamins, activate the

microbial activity in the plant rhizosphere. Ultrastructural studies of the rhizosphere show that the root surface is coated with an amorphous mucilage in which the bacteria are embedded (Campbell and Rovira, 1973). The mucilage matrix (mucigel) is about 20 $\mu$ m thick (Foster and Rovira, 1978). Rhizobia with polar or peritrichous flagella are attracted to the roots of the leguminous plants by chemotaxis. The rhizobia are found within the mucilaginous layer covering the root shortly after their inoculation (Quispel, 1974).

A certain degree of specificity exists between species of legumes and strains of Rhizobium, i.e., a particular strain of Rhizobium infects only certain species of legumes but not others. This specificity leads to the phenomenon of mutual recognition by the Rhizobium strain and the legume species involved. The phenomenon of recognition is mostly, but not always, attributed to plant surface glycoproteins called lectins, that bind to carbohydrates of the rhizobial capsule. Lectins are considered to be responsible for the specific attachment of compatible rhizobial cells on the root hairs and act as a molecular bridge between the common or cross-reactive antigens of the roots and the Rhizobium cells (Dazzo and Hubbell, 1975). The host specificity is therefore partly explained by preferential binding of its homologous Rhizobium strains by specific lectins. The ability of rhizobial cells to develop lectin receptors depends on the growth conditions of the bacteria (Bhuvaneswari and Bauer, 1978). In many cases there is a correlation between lectin binding and Rhizobium infectivity (Bohlool and Schmidt, 1974), even though there are some negative results concerning lectin-mediated recognition. Recognition may not be a single step but the cumulative

effect of a series of signals and receptor interactions with lectin as the first step (Sharifi, 1984).

Once Rhizobium recognizes the host, infection usually occurs through the root hairs. In soybean, the infection is restricted to the zone just below the smallest emerging root hairs and above the region of rapid elongation of the root (Bhuvaneswari *et al.*, 1980). In plants which are not infected through root hairs, such as peanuts and in aquatic plants lacking root hairs, the Rhizobium infection occurs at the lateral root branches or between two epidermal cells (Chandler, 1978). The site of the infection is dependent on the cell wall structure and the rhizobial preference of the point of attachment. The root hair cell wall has two distinct primary and secondary layers. The primary layer is continuous over the entire hair and consists mainly of pectic substances and hemicellulose and, to a lesser extent, cellulose fibrils which are arranged in a random network at the tip of the root hair. The secondary layer is laid inside the primary layer away from the root tip with cellulose fibrils running parallel to the axis of the root hair, suggesting that the root hair is weaker at the tip during development and hence facilitates rhizobial penetration through developing and pre-emerging root hairs. In plants that are invaded at the base of lateral root branches, the infection site is essentially a wound where the cuticle and epidermal cells are injured during the course of normal lateral root growth (Bidwell, 1979). In case of infection between the two epidermal cells, the pectic substances filling the gap between the cells are easily degraded by the pectolytic enzymes. A low level of pectolytic enzymes is detectable in free-living rhizobia (Hubbell *et al.*, 1978; Verma *et al.*, 1978b).

Pectinase activity also increases in the infected roots, but the involvement of cell wall hydrolyzing enzymes in the process of rhizobial infection remains unsolved (Verma, 1982).

The curling of the root hair is the first indication of infection by Rhizobium. Infection occurs more frequently in curled root hairs (Bauer, 1981), but curling is not essential for infection. In the process of curling, the Rhizobium becomes entrapped in the folds and pockets, enabling the accumulation of high concentrations of bacterial substances and hydrolytic enzymes essential for infection (Fahraeus and Sahlman, 1977). Electron microscopic examination of the infection site reveals a disintegration of the cell wall. Callaham (1979) suggests that the cell wall is altered by the hydrolytic enzymes. It has been suggested that the Rhizobium cells infecting the roots of the legume induce the production of wall-degrading enzymes in the plant (Verma *et al.*, 1978b).

Another indication of infection is a colorless spot or a swelling at the site of infection and an increase in cytoplasmic streaming of the plant cell. There is also an increase in the size of the nucleus, a prominent nucleolus and a movement of the nucleus to the site of infection within the cell (Bauer, 1981). The growth of the infection thread produced by the Rhizobium cells is directed by the host cell nucleus that doubles in size and precedes the tip of the infection thread (Fahraeus, 1957). The infection thread starts at the points where curling and branching of the hair form an enclosure (Turgeon and Bauer, 1983). The Rhizobium directly penetrates the hair walls by a localized disintegration of the wall material. A new layer of the wall material is deposited around the infection

sites which encloses the rhizobia and this makes the infection thread wall and the hair cell wall a continuous structure. As rhizobia increase in number and volume the tip of the thread is stretched being the most plastic region of the infection thread. The deposition of the wall material around rhizobia is continued, resulting in the elongation of the infection thread. The infection thread normally grows centripetally and invades the cortex.

In plants which lack root hairs, such as peanuts, the infection is initiated in epidermal cells of the roots. In this case, there is no infection thread formation and the access to the central cortical cells is gained from the site where the epidermis is damaged, like the point of the emergence of lateral root branches or through intercellular spaces (Chandler, 1978). The infection and nodulation occur around the base of the emergent lateral roots in peanuts. The rhizobia enter from the intercellular space into the cytoplasm by disintegrating the plant cell wall. In this respect the infection process in peanut is different from that of other legumes. Recently root-hair-like structures have been found to be present at the site of lateral root emergence in nodulating peanuts, but are reported to be absent in non-nodulating mutant varieties (Nambiar *et al.*, 1983). In nodules without infection threads, all the host cells in the region are invaded by rhizobia, whereas uninvaded host cells are common in the nodules with infection thread (Bauer, 1981).

When the infection thread reaches the cortex, the cortical cells begin to divide to form tetraploid nodule tissue, which in turn is invaded by the Rhizobium. Subsequent division of the tetraploid cells is related to the release of

bacteria from the tip of the infection thread leading to the formation of nodules. Rhizobia from the infection thread are released into the cytoplasm of the host cells singly or in small groups surrounded by a membrane, called the peribacteroid membrane envelope, which keeps them separated from the host cytoplasm. The rhizobia are thus considered extracytoplasmic rather than intracytoplasmic. Presumably, the peribacteroid membrane envelope is derived from the host plasma membrane because of their similarity with regard to thickness and staining properties (Robertson, *et al.*, 1978; Verma *et al.*, 1978a). The peribacteroid membrane envelope differs from the host plasma membrane in its lack of cellulose fibrils and its greater permeability to substances being exchanged between the Rhizobium and the plant cytoplasm.

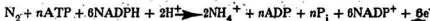
Within the peribacteroid membrane envelope, rhizobia proliferate to the extent that most of the plant cell cytoplasm is occupied. The rhizobia are transformed to a more spherical form, called bacteroids. At this stage they can fix nitrogen and become the nitrogen-fixing organelles of the plant separated from it by a membrane (Verma and Long, 1983). Most of the dry weight of the nodule (25 - 50%) consists of bacteroids (Bergersen, 1974). Bacteroids also have an altered cell wall and plasma membrane composition. They contain more poly- $\beta$ -hydroxybutyric acid granules, glycogen, polyphosphate granules (also known as volutin) and nitrogen reductase than the free living rhizobia (Mackenzie *et al.*, 1973; Bergersen, 1974). Moreover, ribosomes are almost absent from the bacteroids. During transformation into bacteroids, an inhibition of cell wall synthesis occurs, resulting in a thinner and less rigid peptidoglycan wall



(Bergersen and Briggs, 1958; Mackenzie *et al.*, 1973; Napoli and Hubbel, 1975). The cytochrome pattern of the Rhizobium and transformed bacteroids shows that more cytochrome *C* and *P-450* and less cytochrome *a* and *a<sub>3</sub>* are present in the bacteroids as compared with the free living Rhizobium. This pattern shown by the bacteroids is due to low oxygen tension in the host cytoplasm (Trinchant, 1981), which is a suitable environment for the enzyme nitrogenase to fix nitrogen. In general, rhizobia fix nitrogen symbiotically within the nodules, but many Rhizobium strains can also fix nitrogen asymbiotically in pure in vitro culture (Pagan *et al.*, 1975; Bergersen and Gibson, 1977) in the presence of induction medium.

### 1.2.1. Mechanism of Nitrogen Fixation.

The overall process of symbiotic dinitrogen fixation results in the production of ammonia within the bacteroid and can be summarized as:



where  $n = 6.0-6.9$  or  $6.5 \text{ ATP/NH}_4^+$  (Rawsthorne *et al.*, 1980).

In the above biological nitrogen fixation reaction the energy requirement to fix one molecule of ammonia is 355 KJ, whereas for industrial nitrogen fixation the value is approximately 680 KJ. This indicates that the biological nitrogen fixation is twice as efficient as the industrial process. The basic requirements for the reaction to take place are (Bray, 1983):

- the presence of an enzyme nitrogenase,
- a strong reducing agent like NADPH,
- energy source as adenosine triphosphate (ATP),  $Mg^{++}$  ion,
- and low oxygen tension.

Nitrogenase is an enzyme unique to nitrogen-fixing Rhizobium. The production of nitrogenase is coded by the *nif* gene. Nitrogenase is a multi-subunit protein, consisting of two major components, molybdoferredoxin and azoferredoxin of 200,000 and 50,000 daltons, respectively. The larger component, molybdoferredoxin, has four subunits containing molybdenum, non-haem iron and sulfide, and the smaller component azoferredoxin, has only two subunits containing iron and sulfide (Orme-Johnson *et al.*, 1977).

The origin and nature of electron donors vary among the different groups of nitrogen-fixing organisms. In vitro studies show that in nitrogen-fixing Rhizobium NADPH is utilized as a reductant (Wong *et al.*, 1971). In other aerobic nitrogen-fixing microorganisms NADPH is used as a reducing source (Yates, 1977). There have been reports of an  $\text{NADP}^+$ -specific isocitrate dehydrogenase in the bacteroids of the root nodules in legumes, indicating that NADPH may be the electron donor for nitrogenase in vivo (Bray, 1983).

The major energy source in symbiotic nitrogen fixation is ATP. During the process, the mono-magnesium salt of ATP is hydrolyzed to the mono-magnesium salt of ADP and inorganic phosphate. The reduction of other substrates by nitrogenase is also coupled to the hydrolysis of ATP. In vitro studies show that 12 - 15 moles of ATP are consumed for each mole of nitrogen reduced to ammonia. In the consumption of ATP molecules, biological nitrogen fixation is a bioenergetically expensive process. The ATP molecules are supplied by various means; e.g., from the oxidation of respiratory substrates in case of aerobic organisms and via the phosphoroclastic cleavage of pyruvate to acetate in anaerobes.

The enzyme nitrogenase is readily inactivated at high oxygen concentrations (Bergersen, 1971). In order to regulate the oxygen concentration at the site of nitrogen fixation within the nodule, a special type of haemoglobin called leghaemoglobin is synthesized by the host plant (Dilworth, 1969; Verma and Bal, 1976). Leghaemoglobin is a red pigment which gives a characteristic coloration to the nodule. It has a probable location outside the membrane surrounding the bacteroids. Leghaemoglobin has high affinity for oxygen. It aids in the delivery of sufficient oxygen to the bacteroids for their respiratory metabolism, at a concentration level which is not harmful to nitrogenase (Wittenberg *et al.*, 1974). The root nodules can thus be considered as highly specialized compartments providing an environment in which the nitrogen fixation and oxidative metabolism are physiologically compatible.

Ammonia is produced as the first stable end-product by nitrogenase in the bacteroids within the plant root nodules (Kennedy, 1966a, 1966b). Some of the ammonia is utilized by the microorganism for its own growth, but most of it is exported to the host cytosol where it is assimilated (O'Gara and Shanmugam, 1976). The two major ammonium assimilatory enzyme systems are glutamate dehydrogenase which is present in both the host cytosol and the bacteroid, and glutamine synthetase-glutamate synthase which is only active in the bacteroid. Besides these, various other enzymes have been implicated in the assimilatory systems for ammonium ions, such as the bacteroid alanine dehydrogenase (Dunn and Klucas, 1973), and a combination of alanine aminotransferase and glutamate dehydrogenase which is present in the host cell cytosol (Ryan and Fottrell, 1974).

The initial product of ammonia assimilation is glutamine or glutamate. The nitrogenous compounds exported to the host plant have been identified as asparagine, glutamine, aspartate, homoserine and allantoates which are found in proportions which depend on the host legume species (Fig. 1-3). The energy for this process is provided by the breakdown of glucose through the tri-carboxylic acid (TCA) cycle in the host cell.

### 1.2.2. Transport of Fixed Nitrogen.

Most of the information regarding the transport of fixed nitrogen is available from studies in soybean and cowpea. Ureides are present in the nodules of legumes and are the major compounds exported from the nodules to other parts of the host plant (Pate, 1973) (Fig. 1-4). It has been demonstrated that notable quantities of allantoin, a ureide cycle product, is transported from the nodules of soybeans and cowpea (Matsumoto *et al.*, 1977a; 1977b; Herridge *et al.*, 1978), and its amount in soybean plants correlates with the nitrogen fixation rate.

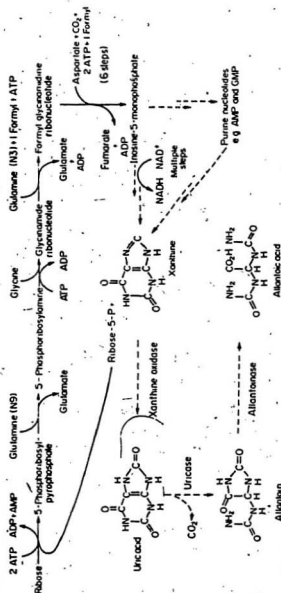
Ureides require fewer carbon atoms to transport the equivalent amount of nitrogen compared to other amino acids which give it an advantage in nodule nitrogen export. The enzyme uricase has also been shown to be more active in the nodules than in the other parts of soybean plant and its activity is associated with the bacteroids (Tajima and Yamamoto, 1977). In cowpea, uricase activity has been shown to be associated with host-cell cytosol and is negligible in the bacteroids (Herridge *et al.*, 1978).

Experimental evidence shows that allantoin is synthesized indirectly from the degradation of purines (Larner, 1971) and that glutamine acts as a nitrogen

**Figure 1-3:** A schematic representation of nodular symbiotic nitrogen assimilation. Aspartate aminotransferase (A), Asparagine synthetase (B), Glutamine synthetase (GS), and Glutamate synthase (GOGAT). (Scott *et al.*, 1976).



**Figure 1-4:** A schematic representation of ureide metabolism.  
(Rawsthorne *et al.*, 1980).





precursor (Fujihara and Yamaguchi, 1978). Since glutamine is one nitrogen-containing product produced through the process of symbiotic nitrogen fixation, it suggests that ureide metabolism is related to nitrogen fixation. Synthesis of allantoin, through xanthine oxidase, generates hydrogen peroxide which is toxic to cellular components. Hydrogen peroxide is degraded to hydrogen and oxygen by the enzyme catalase to remove the intracellular toxicity. The catalase activity has been shown to be correlated with the effectiveness of the bacteroids to fix nitrogen in soybeans (Francis and Alexander, 1972).

Catalase is a marker enzyme for microbodies. Microbodies are a distinct and ubiquitous class of subcellular organelles with specific metabolic functions and are found in all eukaryotes. Microbodies have been characterized and classified on the basis of the enzymes they possess for different metabolic pathways. In addition to specific enzyme complements related to their physiological function, microbodies contain flavin-linked oxidases which generate hydrogen peroxide and a catalase which degrades it (Hall, 1983; Hall *et al.*, 1974; Schnarrenberger and Fock, 1976; Tolbert, 1971). The distinguishing general feature of all microbodies that can be recognized in electron micrographs through DAB (3,3'-diaminobenzidine) reaction, is the presence of catalase (Frederick and Newcomb, 1969; Frederick *et al.*, 1975; Virgil, 1969, 1970, 1973). The microbodies are spherical, elongate or dumb-bell shaped and have a diameter ranging between 0.2 - 1.5  $\mu\text{m}$ . They are bound by a single, lipid bilayer membrane surrounding an amorphous or granular electron-dense matrix, often having a dense nucleoid.

Although two major classes of microbodies have been recognized in animals,

three types of microbodies have been recognized in plants (Beever, 1979; Tolbert, 1981; Vigil, 1983): (1) peroxisomes which contain catalases and oxidases (originally found in mammalian systems and were named after their peroxidative release of  $^{14}\text{CO}_2$  from radioactive  $\text{H}^{14}\text{COOH}$ ); (2) glyoxysomes which contain part or all of the enzymes of the glyoxylate cycle, in addition to the catalases and oxidases (generally found in the endosperms of plant seeds); (3) non-specialized microbodies. Peroxisomes and glyoxysomes are both structurally and functionally related and only differ quantitatively in terms of their enzyme content (Thorpe, 1984).

Plant glyoxysomes also contain all of the enzymes for  $\beta$ -oxidation and a complete sequence of enzymes for the glyoxylate cycle, where (in the conversion of fats to sucrose) lipase acts as the first enzyme for the conversion of lipids to fatty acids (Breidenbach *et al.*, 1968; Cooper, 1971; Cooper and Beever, 1969; Hutton and Stumpf, 1969). These types of microbodies are usually found in germinating fatty seeds (Trelease, 1984).

The second specialized form of plant microbodies, the peroxisomes, found in leaves associated with chloroplasts (Tolbert *et al.*, 1968; 1969), contain high levels of glycolate oxidase, hydroxypyruvate reductase and transaminases, accounting for their role in photorespiration (Tolbert, 1971). They also contain catalase and uricase (Huang and Beever, 1973).

Non-specialized microbodies are found in a variety of plant tissues. The major enzyme constituents of these microbodies are catalase, uricase and glycolate

oxidase. The only known functions of the non-specialized microbodies are in purine catabolism and the detoxification of hydrogen peroxide (Huang and Beevers, 1971).

Overall, the microbodies are responsible for the protection of cells from the toxicity of high oxygen concentrations, the gluconeogenic conversion of fats to sugars, the formation of glycine and serine, compartmentalization of purine and pyrimidine catabolism and D-amino acid destruction and regulation of growth in plants by consumption of excess reducing power.

### 1.3. Arachis hypogaea L. - Bradyrhizobium Interaction.

#### 1.3.1. Taxonomy, Distribution and Importance of A. hypogaea.

Arachis hypogaea L., commonly known as peanut or groundnut, belongs to the sub-family *Papilionoideae* of the family *Fabaceae*. It has about 19 species which usually occur in tropical and subtropical regions. Peanut is an annual or perennial herb and has a well-developed tap-root system with many lateral roots emerging from the hypocotyl and aerial branches. The roots are soft, cylindrical and lack root hairs. Recently root-hair-like structures have been found by Nambiar *et al.* (1983). The primary root grows to a depth of 90 - 120cm with an extensive net of young roots mainly produced at a depth of 10 - 25cm.

Peanut is an economically important crop, and native to South America. It is the only plant species now under cultivation which is not found in the wild state. The crop yield varies from 742 kg/ha to 4400 kg/ha (Duke, 1981). It is a warm season legume and grows well with 50 - 100cm of rainfall per year in well

drained, friable, loam soil with a pH of 5.0-6.5, containing sufficient amounts of phosphates, potassium and calcium (Allen and Allen, 1981).

The commercial importance of peanuts is multifold and it ranks second to soybeans as a source of high quality edible vegetable oil. Peanuts have easily digestible seeds with a high nutritive value. The seed is a rich source of vitamin B-complex, especially thiamin, riboflavin and nicotinic acid. It contains 14 - 24% protein, 60% carbohydrate and 6 - 12% oil (Haq, 1983).

### 1.3.2. Bradyrhizobium Species and the Induction of Nitrogen-Fixing Root Nodules.

Nitrogen assimilation in peanuts is the direct result of symbiotic nitrogen fixation by specific Bradyrhizobium spp. which induce effective root nodules in the plant. Bradyrhizobium is a gram negative bacteria which belongs to the family Rhizobiaceae and is slow growing. Some strains of Bradyrhizobium, called cross-inoculating strains, can nodulate different varieties of peanuts as well as different legume species.

In peanuts rhizobia enter through the ruptured sites of emerging lateral roots instead of through root hairs, and the nodules appear on the roots at about the time the third set of leaves are being formed (Allen and Allen, 1981). The peanut nodules are morphologically simple, being smooth and spherical with a diameter of about 1 - 5 mm. They have a broad basal connection and are mostly clustered on the tap root. In very small newly differentiating nodules of peanuts, the rhizobia are mostly found in the intercellular spaces showing an invagination

into the host cell. They are then released into the host cells and are enclosed in peribacteroidal membrane envelopes (Bal *et al.*, 1985). The rhizobia within the nodule cells undergo considerable morphological changes resulting in the formation of giant spherical bacteroids (Staphorst and Strijdom, 1972; Van Rensburg *et al.*, 1973). Before differentiating into nitrogen-fixing bacteroids, the rhizobia shed off their outer membranes (cell wall) and replace them with a new outer membrane (Bal *et al.*, 1985). The enzyme, nitrogenase, becomes active in differentiated bacteroids and starts fixing nitrogen which is transported out into the host cell as ammonium ions and from there to other parts of peanut. Although it is not known in which form fixed nitrogen is transported, the presence of high levels of 4-methyleneglutamine in the xylem of mature peanut plants suggests that this amino acid may be one of the major nitrogen carriers from the roots to other parts of the plant (Winter *et al.*, 1981).

#### **1.4. Comparative Study of Peanuts and other Related Nodule-Producing Legumes.**

Cytologically, the bacteroidal zone in the nodule of peanuts features some unique properties. It has been shown that the cells are diploid (Kodama, 1967) rather than tetraploid as observed in other nodulating legume species, and that the infection is not accompanied by host cell hypertrophy. Also, because of the absence of an infection thread there is a complete infestation of the newly-formed inner tissue by rhizobia through passive transmission. Comparative histological studies show that peanut root nodules have a much thinner and uniform outer cortex with an inner spherical bacteroidal zone totally infected with bacteroids, as

compared with cowpea where the cortex is much thicker, bacteroidal zone somewhat indented and is represented by both infected as well as uninfected cells (Sen and Weaver, 1984a). The cells containing bacteroids of peanuts are isodiametrical and uniform in size with a central vacuole and a nucleus surrounded by tightly arranged bacteroids. The number of bacteroids is much lower in peanut nodules than in cowpea but the peanut bacteroids are 3.3 - 4.5 times larger, more spherical and one and a half to three-times more effective in fixing nitrogen than are cowpea bacteroids (Sen and Weaver, 1980; 1984a). In these comparative studies, the same strain of rhizobia (32H1) was used to infect the two different hosts.

The peanut bacteroids produced by Bradyrhizobium spp. are morphologically very distinct from those in cowpea as well as in soybean. In peanuts they are enlarged and spherical, have an inner and an outer bacteroidal membrane and are enclosed singly within the peribacteroidal membrane envelope which separates them from the plant cytosol. The cowpea bacteroids, transformed from the same strain of rhizobia, are rod-shaped, branched and smaller in size. Usually only one bacteroid is observed in each membrane envelope, but occasionally several may be present (Sen, Weaver and Bál, 1986). Soybean root nodules have bacteroids which are somewhat small and cylindrical (Newcomb and Tandon, 1981a).

The anatomies of soybean and cowpea root nodules are very similar. Both contain an outer cortical layer which is several cells thick. Inside the nodule is a central zone of enlarged cells infected with rhizobia as well as many smaller

uninfected cells interspersed among the infected ones (Bergersen and Goodchild, 1973; Newcomb, 1981). It has also been shown that at early stages of nodule development the uninfected (interstitial) cells of the soybean root nodules undergo considerable ultrastructural changes, reflected in the enlargement of microbodies (about 60 times) and the proliferation of tubular endoplasmic reticulum. These microbodies are absent or very much reduced in size and number in the infected cells of soybean root nodules (Newcomb and Tandon, 1981a; 1981b; Newcomb *et al.*, 1985). The microbodies in the uninfected cells of soybean root nodules contain most of the enzymes of ureide metabolism like uricase, catalase and allantoinase and are therefore involved in assimilating symbiotically-fixed nitrogen as ureides (Newcomb *et al.*, 1985 and Hanks *et al.*, 1983). Cowpea root nodules, like soybean, have uninfected cells interspersed among the infected ones and assimilate fixed nitrogen as ureides. However, both infected and uninfected cells in cowpea possess the necessary enzyme complement for ureide formation (Shelp *et al.*, 1983). Moreover, the effectiveness of nitrogen fixation in soybeans and other nitrogen-fixing legumes is correlated with high catalase activity (Francis and Alexander, 1972). This catalase may be involved in the detoxification of hydrogen peroxide which is produced during the synthesis of allantoin (a metabolite of ureide metabolism) (Rawsthorne *et al.*, 1980).

Peanut root nodules have a unique anatomy which differentiates them from other nodule-producing legumes (Sen and Weaver, 1984a; Sen, Weaver and Bal, 1986). Peanut root nodules, unlike cowpea and soybean, have been shown to have a central darker zone consisting mainly of infected cells highly infested with

bacteroids. There are very few uninfected cells interspersed among the infected ones, except for rays of cortical cells between infected masses of cells. Within the infected cells of the peanut root nodules the bacteroids are large and spherical occupying a major portion of the host cell. In contrast bacteroids of cowpea and soybean are usually small and cylindrical, occupying a lesser area of the host cell. The higher rates of symbiotic nitrogen fixation in peanuts compared with soybean and cowpea are thought to be related to structural differences in the nodules and bacteroids, and also to the type of interaction between the bacteroids and the host plant (Sen and Weaver, 1984a; Sen, Weaver and Bal, 1986).

The unique anatomy of peanut root nodules and their characteristic bacteroid specialization which results in high rates of nitrogen fixation, compared with cowpea, soybean and other nitrogen-fixing legumes, has prompted a detailed study of the ultrastructure and biochemistry of peanut root nodules.

The primary aim of this research project has been to examine the ultrastructural and enzymatic (nitrogenase and chitinase) aspects of peanut root nodules which may be important to their high nitrogen-fixing ability.



## Chapter 2

### MATERIALS AND METHODS

#### 2.1. Bradyrhizobium sp. and its Cultural Conditions.

Bradyrhizobium sp. 32H1 (Nitragin Co., Milwaukee, Wisconsin, USA) was used as a specific strain to produce effective root nodules in Arachis hypogaea L. Var. Jumbo virginia.

Bradyrhizobium sp. 32H1 was maintained on yeast extract-mannitol agar. It was cultured in yeast extract-mannitol broth containing mannitol, 10g;  $K_2HPO_4$ , 0.5g;  $MgSO_4 \times 7H_2O$ , 0.2g; NaCl, 0.1g; yeast extract, 0.4g; distilled water, 1 liter; pH, 6.8 - 7.0 (Vincent, 1970) with constant shaking (140 - 150 rpm) at 28°C in an orbit Environ-shaker, Lab-line Instruments Inc.

To obtain an in vitro culture of differentiated bacteroids, 32H1 was also grown in induction medium LNB5-GAS (Van Brussel *et al.*, 1979) which contained glutamine, 300mg; succinate, 6.75g; arabinose, 3.7533g;  $MgSO_4$ , 250mg;  $NaH_2PO_4 \times H_2O$ , 150mg;  $CaCl_2 \times 2 H_2O$ , 150mg; myo-inositol, 100mg; sucrose, 30g; thiamine, 10mg; niacin, 1mg; pyridoxin-HCl, 1mg; and trace elements: Fe, 28mg;  $MnSO_4$ , 10mg;  $H_3BO_3$ , 3mg;  $ZnSO_4$ , 2mg;  $Na_2MoO_4$ , 0.25mg;  $CuSO_4$ , 0.025mg;  $CoCl_2$ , 0.025mg; KI, 0.78mg in 1 liter of distilled water.

## 2.2. Arachis hypogaea L. and its Growth Conditions.

The seeds of Jumbo virginia were obtained from W. Atlee Barpee Co., Warminster, Pennsylvania, USA. The seeds were surface sterilized by rinsing them with 95% ethyl alcohol for 1 - 2 minutes, immersing them in 0.2%  $\text{HgCl}_2$  for 5 minutes, then thoroughly washing in sterilized distilled water (Vincent, 1970). These surface-sterilized seeds were germinated on moist paper towel for 5 - 7 days, inoculated with broth culture of 32H1 and sown in vermiculite, moistened with a nitrogen-free nutrient solution (Vincent, 1970). All steps were carried out under sterile conditions. The seeds were grown in an environmental chamber (Controlled Environments Ltd.) under controlled conditions of light, temperature and humidity. The photoperiod was kept as 16 hours light and 8 hours dark. Day temperature was kept at 27°C and the night temperature at 22°C, day and night humidities were 70% and 50% respectively.

## 2.3. Light and Electron Microscopy of A. hypogaea Root Nodules.

### 2.3.1. Light Microscopy.

Healthy root nodules from three to four week old peanut plants were frozen at -25°C in a cryo-cut microtome (American Optical Corporation). After complete freezing, 20 / 30  $\mu\text{m}$  thick sections were cut and stained with alcoholic safranin and 1% fast green. The sections were rinsed with distilled water and passed through a series of alcohol grades and xylene, and then mounted in permount (Fisher Scientific Co.). Observations were made with a Zeiss photomicroscope.

### 2.3.2. Histochemical Test for Lipids.

Fresh and healthy root nodules from three to four week old peanut plants were used to test for the presence of lipids in the nodule cells. The nodules were first thoroughly washed with distilled water and then squashed on a glass slide. The squashed nodules were stained with Sudan-III and immediately mounted with a cover slip and sealed with paraffin to prevent drying. The preparations were observed with a Zeiss photomicroscope.

### 2.3.3. Electron Microscopy.

Thick hand sections (80 - 100 $\mu$ m) of fresh and healthy peanut root nodules of different developmental stages, (3-8 weeks) were cut and fixed in a mixture of paraformaldehyde and glutaraldehyde in 0.1M phosphate buffer (pH 7.2) (Karnovsky, 1965) and incubated for 60 minutes at 0-4°C. After 1 hour, for at least 15 minutes, the sections were washed thoroughly with 0.1M phosphate buffer (pH 7.2) at 0-4°C. The sections were then postfixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.2) for 60 minutes at 0-4°C. The sections were then washed with phosphate buffer and dehydrated by passing them through a series of ethanol (35%, 50%, 70%, 80%, 95% & absolute). The exposure was 5 minutes in each concentration, and at least 60 minutes in absolute ethanol, which was changed every 20 minutes. After dehydration, the sections were transferred to a 1:1 (V/V) mixture of absolute alcohol and Spurr's embedding resin (Spurr, 1969) and left under vacuum for 30 minutes. The amount of resin in the mixture was doubled (1:2) and tripled (1:3) every 30 minutes. The sections were then transferred to 100% resin and kept overnight under vacuum. The sections were

embedded with resin in capsules or blocks which were polymerized at 70°C for at least 8 hours.

Ultra-thin plastic sections were cut with the help of a Sorvall MT-1 ultra-microtome poststained with lead citrate and uranyl acetate and observed under a Zeiss 109 transmission electron microscope. Plastic sections 0.5 - 1.0  $\mu$ m thick, stained with 1% toluidine blue in 1% sodium borate, were also observed under a Zeiss photomicroscope.

## 2.4. Nitrogenase Assay by Acetylene ( $C_2H_2$ ) Reduction Technique.

Peanut root nodules at different developmental stages (3-8 weeks old) were assayed for nitrogenase activity following the method of Hardy *et al.* (1968). Fresh root nodules were picked and placed in 13 ml vacuum tubes with air tight rubber stoppers. Using a pressure lock gas syringe (Precision Sampling Corporation), 0.1 atmosphere of air was then replaced by 0.1 atmosphere of acetylene (freshly made up in the lab.). The nodules were incubated with acetylene for 3 hours at room temperature. Two controls were used for the assay: (1) without any nodules, containing 0.1 atmosphere of pure acetylene only; (2) root nodules without acetylene. After incubation, 1 ml of gas sample was taken from each tube and injected individually into a Basic<sup>TM</sup> Gas Chromatograph-GC 9700 (Carle Instruments Inc.) with a hydrogen flame ionization detector and helium as a carrier gas. The unchanged acetylene and the ethylene produced were indicated as peak heights on an Omni-scribe recorder (Houston Instruments). The nitrogenase activity was calculated using standard curves for acetylene and

ethylene and expressed as n mols/hr/mg nodule wt. The nitrogenase activity assays were repeated on five different batches of same age group peanut plants and standard deviation from the mean values was calculated.

## **2.5. Fractionation of Peanut Root Nodule Components and**

### **Isolation of Bacteroids.**

Peanut root nodules were fractionated at different stages of their development (3-8 weeks). Fresh and healthy root nodules were picked and kept at 0-4°C (on ice). They were weighed and washed with cold deionized water (0-4°C) to remove any particles of vermiculite attached to them. For each gram of nodules, 2 - 4ml of cold 50mM phosphate buffer (pH 7.0) was added and the nodules were crushed and homogenized very gently using a mortar and pestle. The homogenate was diluted 40 times with 50mM phosphate buffer (pH. 7.0) and then centrifuged at 265 x g for 10 minutes at 0-4°C. The pellet containing plant cell wall debris was discarded and an aliquot of the supernatant (total nodule extract or whole homogenate) was retained. The rest of the supernatant was centrifuged at 14,000 x g for 10 minutes at 4°C in a Sorvall RC-5 Super Speed Centrifuge. The resultant pellet contained the bacteroids and the supernatant contained plant cytosol and organelles.

## **2.6. Catalase Assay of Different Fractions of Root Nodules.**

Different fractions of peanut root nodules, namely whole homogenate fraction (consisting of bacteroids and plant cell cytosol and organelles), bacteroid fraction and the plant cytosol and organelle fraction were assayed for their catalase activity at different stages of the nodule development (3-8 weeks old) by

the spectrophotometric method of Beers and Sizer (1952). The amount of protein present in different fractions was determined by Lowry's method (Lowry *et al.*, 1951).

In the catalase assay procedure, 2ml of the fraction (whole homogenate fraction, bacteroid fraction or plant cytosol and organelle fraction) made in 50mM phosphate buffer (pH 7.0) was taken in a 3ml cuvette. To this sample 1ml of 59mM  $H_2O_2$  in 50mM phosphate buffer (pH 7.0) was added just before starting the reaction (zero time). In a control reaction, distilled water was used instead of the enzyme mixture. The reactants were mixed thoroughly and the change in absorbance was recorded at 10 second intervals for 70 seconds at 240nm on a Shimadzu UV-260 double beam spectrophotometer. The specific activity of catalase was calculated as units/mg of proteins with the formula:

$$= \frac{\text{Change in absorbance/minute} \times 1,000}{43.8 \times \text{mg protein/ml of reaction mixture}}$$

Where 43.8 = Molar Absorbance Index For  $H_2O_2$ , and one enzyme unit is equal to one micromole of hydrogen peroxide decomposed per minute (Worthington Enzyme Manual, 1972).

The catalase activity assays were repeated on all fractions of peanut root nodules from four different batches of same age group plants and standard deviation from the mean values was calculated.

## 2.7. Catalase Assay of In vitro Grown Cultures of 32H1.

Bradyrhizobium strain 32H1 was grown in yeast extract-mannitol broth for 8 - 12 days. It was centrifuged at  $14,000 \times g$  in a Sorvall RC-5 Super Speed Centrifuge at  $4^{\circ}\text{C}$  for 15 - 20 minutes to obtain a hard pellet of 32H1. A small portion of the pellet was suspended in 50mM phosphate buffer (pH 7.0) and set aside for catalase assay. The rest of the pellet was made into a thick paste with 50mM phosphate buffer (pH 7.0). After freezing at  $-20^{\circ}\text{C}$  under dry ice it was subjected to high hydraulic pressure in a Carver Laboratory Press - model C (Fred S. Carver Inc., USA). The broken rhizobia were recentrifuged (at the same speed and temperature indicated above) for 5 - 10 minutes. The supernatant obtained from broken 32H1 cells and the aliquot of intact bacterial suspension which was set aside (see above) were subjected to catalase assay as described in section 2.6, and the specific activity was calculated by measuring the amount of protein/ml. The catalase assays were repeated on three different batches of in vitro grown 32H1 and standard deviation was calculated.

## 2.8. Electron Microscopy of Isolated Bacteroids.

The isolated bacteroids obtained from mature (35 days old) peanut root nodules were also processed for electron microscopy. The bacteroid pellet was washed with cold 50 mM phosphate buffer (pH 7.0) and centrifuged at  $14,000 \times g$  in a Sorvall RC-5 Super Speed Centrifuge at  $4^{\circ}\text{C}$  for 10 - 15 minutes.

The pellet was cut into small chunks and fixed in a mixture of paraformaldehyde and glutaraldehyde fixative (Karnovsky, 1965) for 60 minutes at  $0-4^{\circ}\text{C}$ . They were then washed with ice cold 50mM phosphate buffer (pH 7.0).

A fraction of the pellet was also treated with 0.2% NP40 (detergent) in 50mM phosphate buffer (pH 7.0) for 2 hours at 4°C and processed for electron microscopy as described earlier.

## 2.9. Cytochemical Localisation of Catalase by Electron Microscopy.

Cytochemical localization of catalase in peanut root nodules at different stages of nodule development (3-8 weeks) was done using the diaminobenzidine (DAB) reaction (Graham and Karnovsky, 1966) following the procedure described by Frederick and Newcomb (1989).

Thick hand sections (80 - 100µm) of fresh and healthy peanut root nodules were sliced in the presence of a mixture of paraformaldehyde and glutaraldehyde fixative in 50mM potassium-phosphate buffer (pH 6.8) (Karnovsky, 1965). The sections were kept in the fixative for 60 minutes at 0-4°C. They were then washed thoroughly with 50mM potassium-phosphate buffer (pH 6.8) for at least 15 - 20 minutes at 0-4°C.

The fixed and washed sections were pre-incubated for 60 minutes at 0-4°C in a pre-incubation mixture containing 10mg DAB (3,3'-diaminobenzidine - tetrahydrochloride) and 5ml 2-amino-2-methyl-1,3-propanediol 50mM buffer at pH 9.5 - 10.0. Pre-incubation was followed by incubation of the sections at 37°C for 60 minutes with gentle shaking in a media containing 10mg DAB, 5.0ml propanediol buffer and 0.1ml of 3% H<sub>2</sub>O<sub>2</sub> at pH 9.0.

After incubation the sections were washed with buffer and post-fixed in 2%



osmium tetroxide in 50mM potassium-phosphate buffer (pH. 6.8) for 2 hours. They were then processed for electron microscopy following routine procedures.

Five different controls were used for DAB reaction: To inactivate the enzyme, sections were (1) pre-boiled in the buffer, after fixation and subsequent washing, (2) 0.1M KCN and (3) 0.02M 3-amino-1,2,4-triazole (a competitive inhibitor) were added to the pre-incubation and incubation media. The sections were also processed (4) without DAB in both pre-incubation as well as incubation media, and incubation was carried out (5) without the substrate hydrogen peroxide.

## Chapter 3

# RESULTS

### 3.1. Growth Pattern of Bradyrhizobium and A. hypogaea.

Bradyrhizobium sp. 32H1 cultured in yeast extract-mannitol broth showed peak growth in 8-9 days at an optimum temperature of 28°C and pH 6.8.

A. hypogaea seeds showed the first signs of producing a radicle through the testa on the third day of germination. The lateral roots emerged in 5-7 days time and when inoculated and sown showed their first leaves emerging through the vermiculite in another 5-7 days. The peanut plants (Fig. 3-1) started forming root nodules about 2 weeks after inoculation. The nodules increased in size and were fully developed (with respect to their nitrogen-fixing ability and rhizobial differentiation into bacteroids) in 4-5 weeks time at a size of about 2-3mm in diameter (Fig. 3-2). The plants remained healthy in vermiculite for a period of 7-8 weeks with a subsequent yellowing and wilting of the leaves after 8 weeks.

### 3.2. Nitrogenase Activity.

Nitrogenase activity was very low in newly differentiating nodules. There was a steady increase in the rate of nitrogen fixation along with the maturation of the nodules.

Figure 3-1: A mature plant of A. hypogaea L. Var. Jumbo virginia grown under controlled light, temperature & humidity conditions.



Figure 3-2: A. hypogaea L. Var. Jumbo virginia root system with nodules induced by Bradyrhizobium sp. 32H1.



The activity increased considerably after the second week, reaching its maximum at about the 5th week after inoculation (Fig. 3-3). The nitrogenase activity then declined with the increase in age of the nodules and dropped about 80% from the peak in 7 week old root nodules.

### 3.3. Catalase Activity.

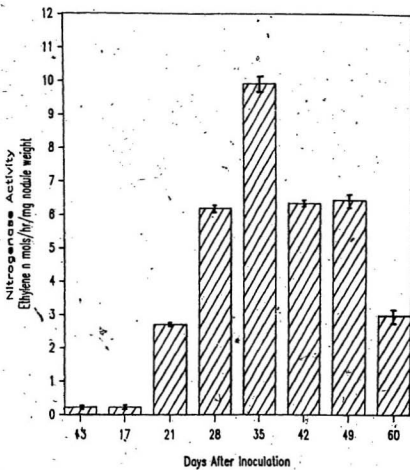
Different fractions (whole homogenate, fraction, bacteroid fraction, and plant cytosol and organelle fraction) of peanut root nodules assayed for catalase activity at different stages of nodule development showed a trend very similar to that seen for nitrogenase activity (Fig. 3-4).

The isolated bacteroid fraction showed higher specific activity of catalase compared to the whole homogenate fraction, the plant cytosol and the fraction carrying organelles. The catalase activity of the nodules increased with the increase in the age of the plant. In newly differentiating root nodules (around 3 weeks) the activity was low. Activity increased with maturity and reached a maximum at 5 weeks. After the 5th week the activity declined to low values in 7 week old root nodules.

The in vitro grown 32H1 showed negligible or no catalase activity in intact bacteria but the supernatant from broken bacteria gave a high value of specific activity for catalase (Table 3-1).

**Figure 3-3:** Nitrogenase (Acetylene reduction) activity in root nodules of A. hypogaea, assayed at different days after inoculation. Mean values with standard deviation are plotted ( $N=5$ ).





**Figure 3-4:** Catalase activity in different fractions of root nodules of A. hypogaea, assayed at different days after inoculation. Mean values with standard deviation are plotted (N=4).

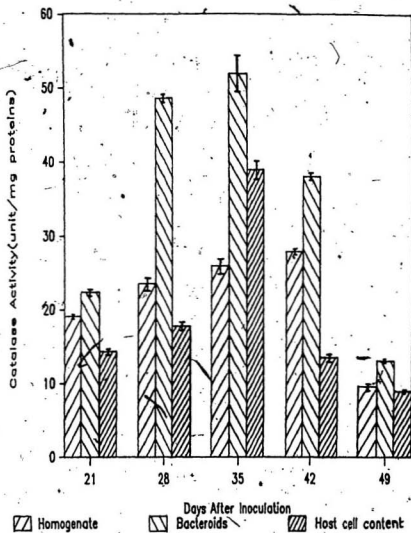


Table 3-1: Catalase activity of in vitro grown 32H1.

| <u>Fraction</u>                     | <u>Specific Activity</u> |
|-------------------------------------|--------------------------|
| Intact bacteria                     | $0.030 \pm 0.02$         |
| Supernatant from<br>broken bacteria | $8.419 \pm 0.26$         |

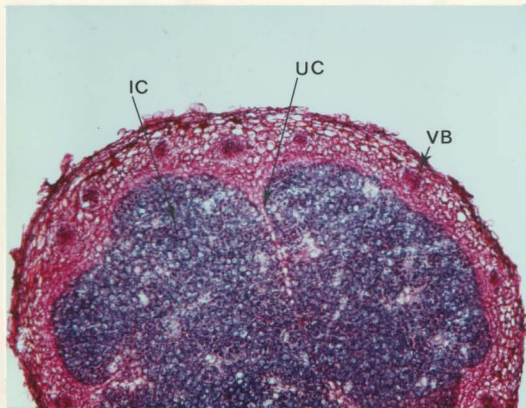
### 3.4. Nodule Anatomy and Ultrastructure.

The peanut root nodule section under the light microscope (Fig. 3-5) showed an inner dense infected zone with cells highly infested by Bradyrhizobium 32H1 and showed no uninfected cells interspersed among the infected cells. The outer cortical region, however, consisted of uninfected cells clearly demarcated from the infected region, being lighter and having uniformly arranged vascular bundles around the central infected region. Rays of uninfected cells traversed the central infected zone, thereby dividing the infected zone into two or three masses of infected cells.

The ultra-thin sections of root nodules, observed under the electron microscope showed characteristic tightly-packed bacteroids occupying a major portion of the host cell cytosol and were arranged around the central vacuole with the cell nucleus adpressed to its side (Fig. 3-6). The bacteroids were much enlarged and spherical in appearance, bounded by an inner and an outer bacteroidal membrane and were enclosed singly in an envelope sac known as the peribacteroidal membrane envelope. The bacteroids contained glycogen, poly- $\beta$ -hydroxybutyric acid granules, ribosomes and a central core of DNA (Fig. 3-7).

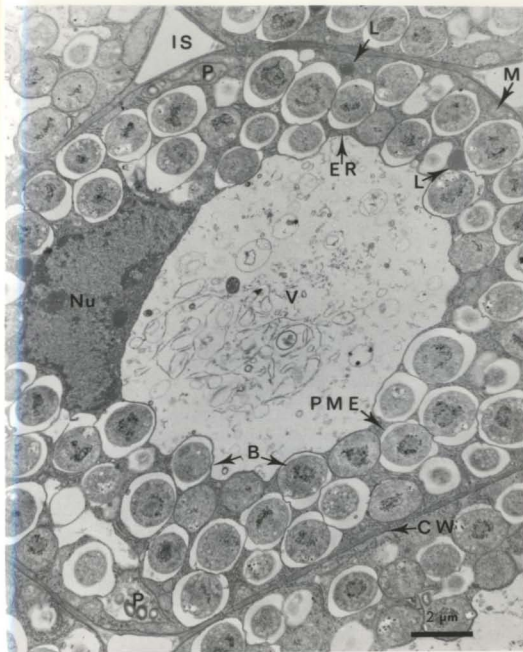
The cytosol of host plant cells contained a number of mitochondria and proplastids located close to the plant cell wall. Fragments of endoplasmic reticulum and lipid bodies could also be found between the peribacteroidal membranes in the cytoplasm.

**Figure 3-5:** Photomicrograph of a frozen section of A. hypogaea root nodule as seen under the light microscope after staining with fast green and safranin. Note the masses of infected cells (IC), rays of uninfected cells (UC) and uniformly arranged vascular bundles (VB) within a thin layer of cortex around the central infected region. (x96).

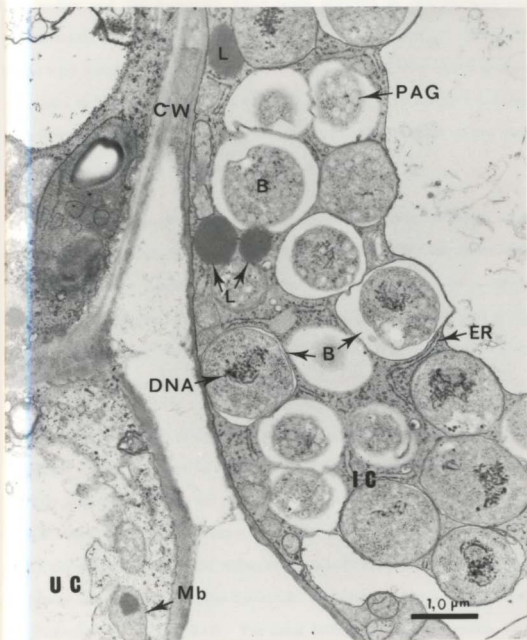


**Figure 3-8:** Ultrastructure of an infected cell of A. hypogaea root nodule showing bacteroid (B), cell wall (CW); endoplasmic reticulum (ER), intracellular space (IS), lipid body (L), mitochondria (M), nucleus (Nu), proplastid (P), peribacteroidal membrane envelope (PME) and cell vacuole (V).





**Figure 3-7:** Ultrastructure of A. hypogaea root nodule cells showing infected cell (IC) with spherical bacteroids (B) containing poly- $\beta$ -hydroxybutyric acid granules (PAG) and a central core of DNA. Endoplasmic reticulum (ER) is seen running between the peribacteroidal membrane envelopes (PME). Also note the presence of lipid bodies (L) and numerous mitochondria (M) near the plant cell wall (CW), and a typical microbody (Mb) in the uninfected cell (UC).



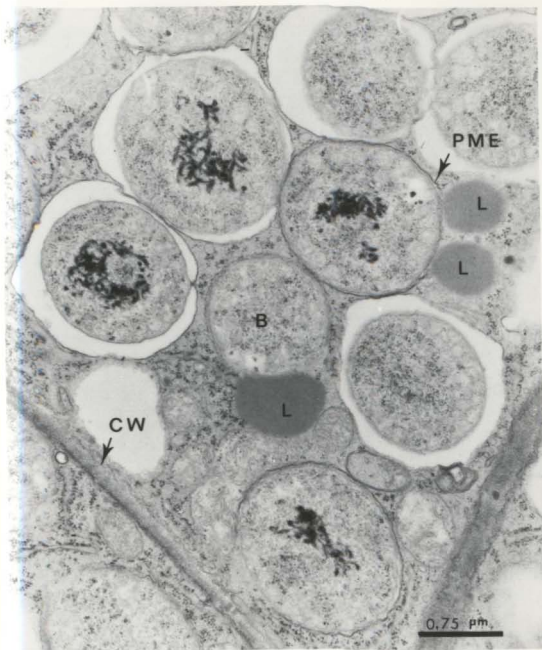
The ultrastructure examination also revealed very close contact between the lipid bodies and the bacteroids which were observed in contact with the peribacteroidal membrane envelope and apparently fusing with it. The lipid bodies were found at all stages of nodule development, but were seen in greater abundance in fully-developed root nodule cells (Fig. 3-8). The squashed nodules, stained with sudan-III, showed the presence of lipid bodies which stained red. They were found in close association with the bacteroids.

The nodule cells also showed the presence of microbodies which were characteristically  $0.2 - 0.75 \mu\text{m}$  in diameter, had a granular to fibrillar matrix with a dense amorphous nucleoid-like structure and were bounded by a single membrane. Microbodies were found in both infected and uninfected cells of the root nodules; the larger ones being in the uninfected cells (Fig. 3-9 & 3-10). They were present at all stages of nodule development.

The microbodies in infected cells were present in close association with the bacteroids. These were seen in close contact with the peribacteroidal membrane envelope (Fig. 3-10). Besides the presence of well-defined microbodies, amorphous electron-dense structures called dense bodies were seen in the infected cells.

These dense bodies were found either at the interface between peribacteroidal membrane envelope and the outer bacteroidal membrane or within the matrix of the bacteroids (Fig. 3-10 & 3-11). The dense bodies, like the microbodies, were found to persist at all stages of nodule development. Even after the removal of peribacteroidal membrane envelope with a detergent (NP40) in the isolated bacteroid fraction, the dense bodies were found to remain attached to the bacteroidal outer membrane (Fig. 3-12).

**Figure 3-8:** Ultrastructure of a mature A. hypogaea root nodule cell showing the close association of lipid bodies with the bacteroids. Note the virtual fusion of lipid body with the bacteroid. Bacteroid (B), cell wall (CW), lipid body (L) and peribacteroidal membrane envelope (PME).



**Figure 3-9:** Ultrastructure of A. hypogaea root nodule showing both infected and uninfected cells and the presence of a typical microbody in the uninfected cell. Bacteroid (B), cell wall (CW), infected cell (IC), mitochondria (M), microbody (Mb), uninfected cell (UC) and cell vacuole (V).

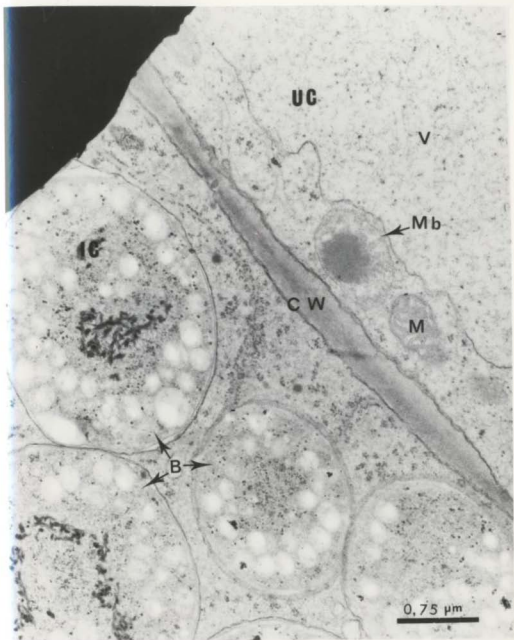
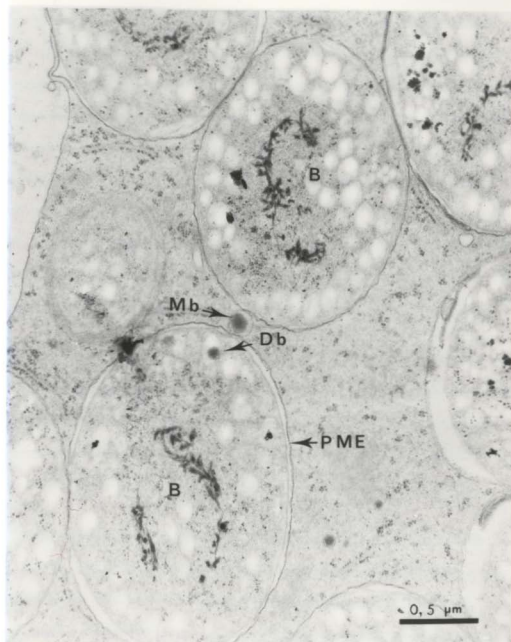




Figure 3-10: Ultrastructure of a mature A. hypogaea root nodule cell showing the presence of both dense body and microbody in close association with the bacteroid (B), dense body (Db), microbody (Mb) and peribacteroidal membrane envelope (PME).



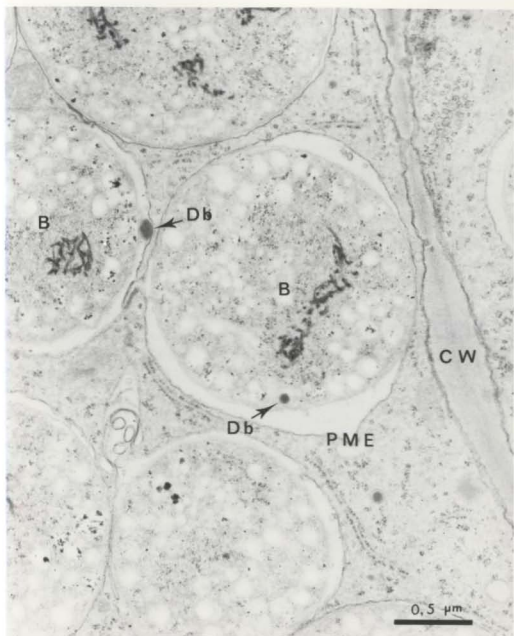
### 3.5. Catalase/Microbodies Localization.

Catalase, the marker enzyme for microbodies, was localized to characterize microbodies within the root nodule cells. Diaminobenzidine (DAB) reaction is specific for the cytochemical localization of catalase. In the presence of hydrogen peroxide and catalase activity, DAB is polymerized, which in turn reacts with osmium to produce osmium-black electron-dense deposits. The electron-dense DAB-osmium complex indicates the presence of catalase activity. DAB-positive reaction products were found in the microbodies, which were surrounded by a single membrane, sometimes appearing discontinuous and there was a nucleoid-type, amorphous, inner electron-dense region, slightly eccentrically placed (Fig. 3-13). Sometimes they appeared as pear-shaped structures, continuous with the endoplasmic reticulum (Fig. 3-14).

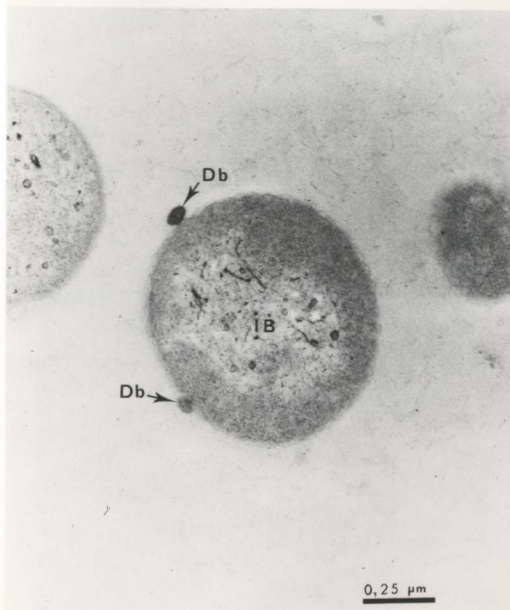
In a mature infected cell, the diaminobenzidine (DAB) reaction revealed the presence of microbodies in abundance (Fig. 3-15) and they were found in close association with the bacteroids showing contact with the peribacteroid membrane envelope. In some sections the outer membrane of the microbodies was seen to be continuous with the peribacteroid membrane envelope (Fig. 3-16).

The osmium-black electron-dense DAB reaction product could also be detected in dense bodies which are dense amorphous structures without any membrane and show their close association with the bacteroids (Fig. 3-17). The dense bodies were found in the interface between the peribacteroid membrane envelope and the outer membrane of the bacteroid (Fig. 3-18). These dense bodies were also present within the matrix of the bacteroids. The dense bodies

**Figure 3-11:** Ultrastructure of a mature *A. hypogaea* root nodule cell showing a close association of dense bodies with the bacteroids (B), cell wall (CW), dense body (Db) and peribacteroidal membrane envelope (PME).



**Figure 3-12:** Ultrastructure of isolated bacteroids from A. hypogaea root nodules showing dense bodies (Db) attached to the outer membrane of the isolated bacteroid (IB).



**Figure 3-13:** Ultrastructure of A. hypogaea root nodule showing an uninfected cell with a typical microbody after DAB reaction. Cell wall (CW), mitochondria (M), microbody (Mb), uninfected cell (UC) and cell vacuole (V).



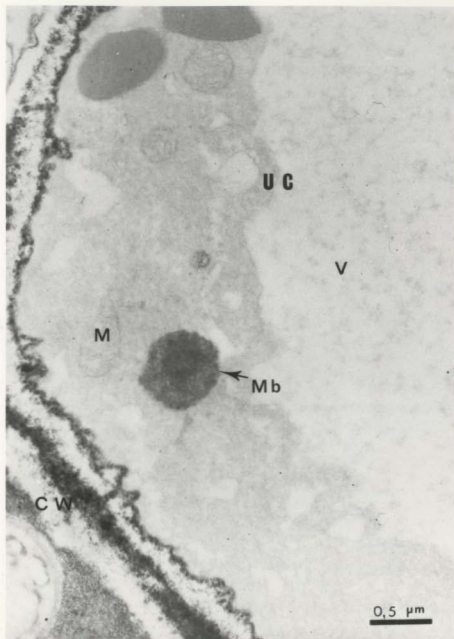


Figure 3-14: Ultrastructure of a mature root nodule cell of A. hypogaea showing a typical pear-shaped microbody through DAB reaction in close contact with the peribacteroidal membrane envelope and endoplasmic reticulum. Bacteroid (B), endoplasmic reticulum (ER), microbody (Mb) and peribacteroidal membrane envelope (PME).

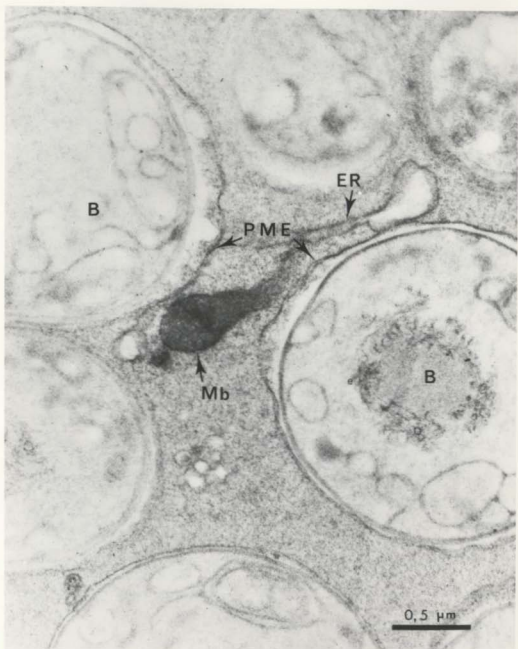
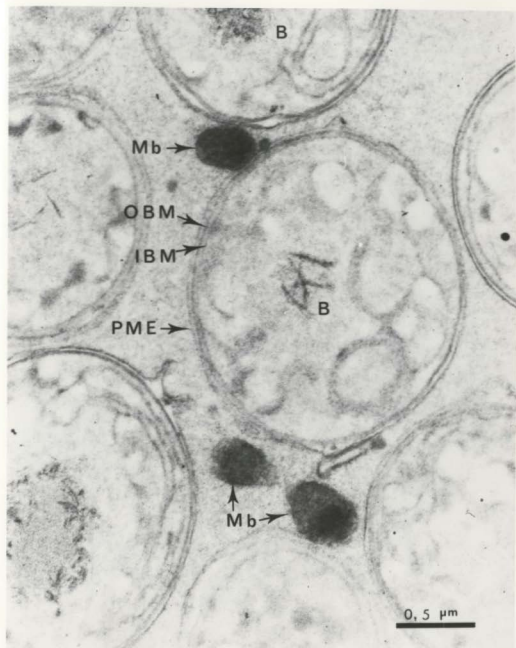
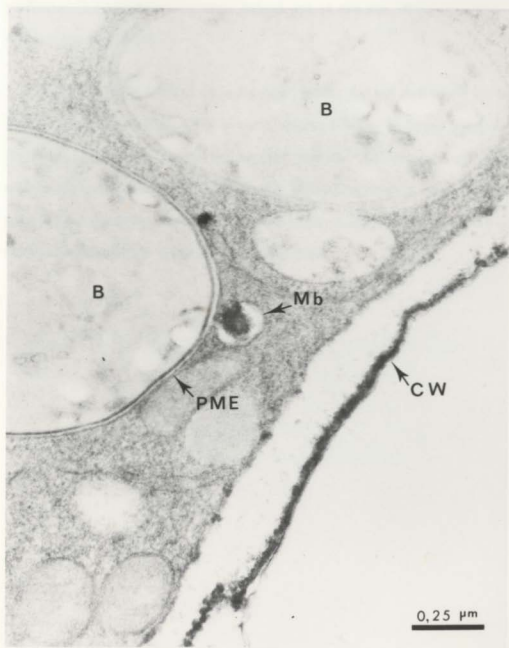


Figure 3-15: Ultrastructure of a mature A. hypogaea root nodule cell showing the abundance of microbodies, the density of reaction product and their close association with the bacteroids through DAB reaction. Also shown are the inner and outer bacteroidal membranes in fully-developed bacteroids (B), inner bacteroidal membrane (IBM), microbody (Mb), outer bacteroidal membrane (OBM) and peribacteroidal membrane envelope (PME).



**Figure 3-16:** Ultrastructure of a mature A. hypogaea root nodule cell showing a continuity of the microbody membrane with the peribacteroidal membrane envelope through DAB reaction. Bacteroid (B), cell-wall (CW), microbody (Mb) and peribacteroidal membrane envelope (PME).

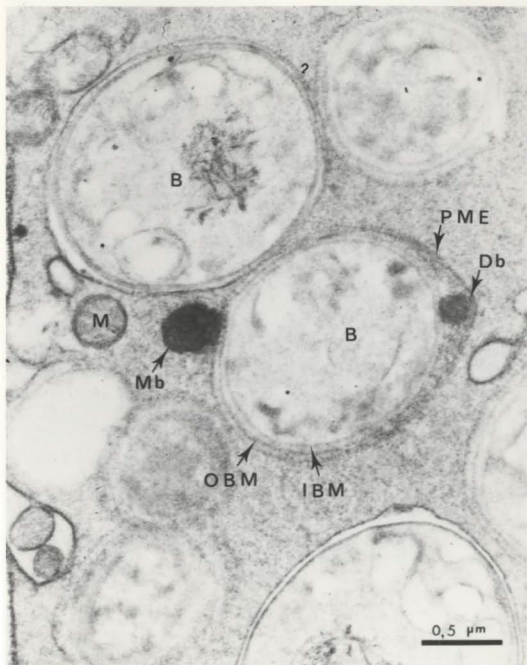


appeared to be pressed against the outer bacteroidal membrane for the peribacteroidal membrane envelope bulged out at the site where dense bodies were present (Fig. 3-19).

The osmiophilic electron dense reaction product was not seen in the controls used for the DAB reaction. The controls without DAB or hydrogen peroxide in the incubation mixture showed no reaction product. KCN-treated and boiled sections showed partial or complete absence of reaction product. 3-Amino-1,2,4-triazole, a competitive inhibitor, also showed the absence of the characteristic reaction product within the root nodules (Fig. 3-20).



**Figure 3-17:** Ultrastructure of a mature A. hypogaea root nodule cell showing the presence of both dense bodies and microbodies and their close association with the bacteroid through DAB reaction. Bacteroid (B), dense body (Db), inner bacteroidal membrane (IBM), mitochondria (M), microbody (Mb), outer bacteroidal membrane (OBM) and peribacteroidal membrane envelope (PME).



**Figure 3-18:** A mature A. hypogaea root nodule cell showing a dense body at the interface between the peribacteroidal membrane envelope and the outer bacteroidal membrane, shows positive DAB reaction. Bacteroid (B), dense body (Db) and peribacteroidal membrane envelope (PME).



**Figure 3-19:** Ultrastructure of a mature A. hypogaea root nodule cell showing ~~the~~ close association of the dense bodies with the bacteroid and characteristic bulging of the peribacteroidal membrane envelope at the site where dense bodies are localized through DAB reaction. Bacteroid (B), dense body (Db) and peribacteroidal membrane envelope (PME).

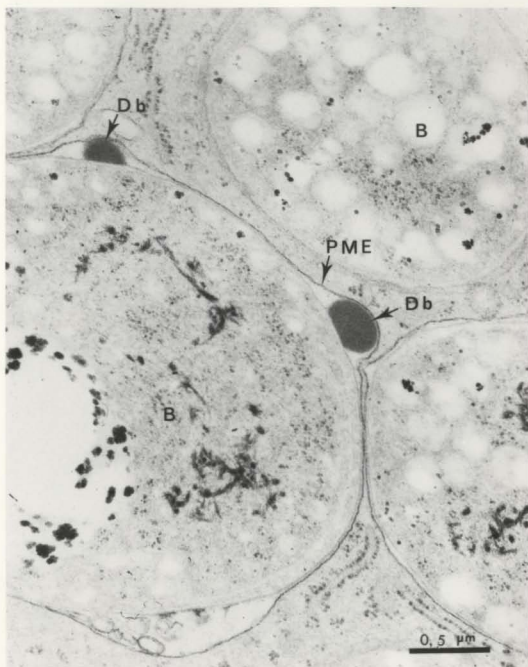
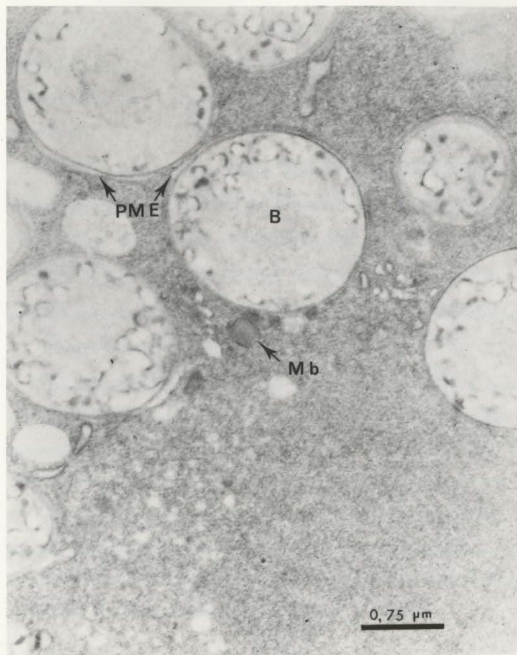


Figure 3-20: Control preparation of an A. hypogaea root nodule cell showing the absence of DAB reaction product in the control incubated in presence of 3-amino-1,2,4-triazole (AT).  
Bacteroid (B), microbody (Mb) and peribacteroid membrane envelope (PME).





## Chapter 4

### DISCUSSION

The main objective of the present study was to examine the interaction between Bradyrhizobium sp. 32H1 and the legume Arachis hypogaea L. Var. Jumbo virginia, in an attempt to understand some of the reasons why their symbiosis results in the production of highly effective nitrogen-fixing nodules.

#### 4.1. Catalase Activity and Nitrogen Fixation ( $C_2H_2$ Reduction) in Peanut Root Nodules.

The catalase activity of peanut root nodules at different stages of nodule development was low in young (2-3 weeks old) differentiating nodules and high in mature (4-5 weeks old) nodules when the activity had reached its maximum. The peak was followed by a decline in enzyme activity along with progressive aging of the nodules. The results also showed higher catalase activity in the bacteroid fraction as compared to the plant cytosol and organelle fraction, indicating that most of the enzyme activity is associated with the bacteroids within the host plant cell and that this activity increases with maturation.

The catalase activity was also assayed in the bacteria grown in culture in order to determine if the enzyme was present in free living asymbiotic form or induced in the nodule during nitrogen fixation and symbiosis. There was no or

negligible activity in the intact bacteria, although the supernatant from the broken bacteria yielded high catalase activity, indicating the presence of the enzyme within the bacterial cells. The trace amounts of activity recorded in intact cells is most likely due to the presence of a few broken bacterial cells. In the transformed bacteroids isolated from the nodule homogenate, most of the activity was observed in intact bacteroids, indicating that the enzyme was probably present on the surface of the bacteroids or was transported onto the surface due to a possible increase in membrane permeability.

The results have shown that small amounts of nitrogen are fixed by young differentiating root nodules. The nitrogen-fixing ability of the plant increases with maturation, reaching its maximum in fully-developed nodules/bacteroids. The decline which follows is attributed to the progressive degradation of the nodule cells and the decline in the number of bacteroids. A similar trend of increased nitrogenase activity of root nodules with maturity along with increased numbers of bacteroids has been reported by Sen and Weaver (1980), in a different cultivar of peanut, namely Tamnut.

The effectiveness of nitrogen fixation in soybean and other nodulating legumes has been shown to correlate with high catalase activity (Francis and Alexander, 1972). In peanuts, there is temporal correlation between catalase activity and nitrogenase activity (acetylene reduction). Both nitrogenase and catalase activities increase with the maturation of the nodules, although catalase is not directly involved in nitrogenase activity per se. The correlation between nitrogen fixation and host cytosol catalase activity is possibly due to an efficient coupling of assimilation and further metabolism of the fixed nitrogen.

Besides producing effective root nodules in peanuts, the strain 32H1 also produces effective root nodules in cowpea. However the nitrogenase activity in peanut root nodules is one and a half to three times higher than in cowpea (Sen and Weaver, 1980; 1981a). The basis for the different rates of nitrogen fixation by the same strain of Bradyrhizobium has been attributed to differences in the morphology of the bacteroids and to the structural organization within the nodules of the two legume species (Sen and Weaver, 1984a; Sen, Weaver and Bal, 1986). The in vitro nitrogenase activity experiments on isolated bacteroids from the two legume species done by Sen and Weaver (1981b, 1984b) also supported the hypothesis that the higher nitrogen-fixing ability of peanuts was related to the unique structural modification of the rhizobial strain 32H1 and to the nodule itself.

#### 4.2. The Root Nodule Anatomy and Ultrastructure.

The root nodules of peanut have unique cytological features which differentiate them from the root nodules of other nitrogen-fixing legumes. There is a large central dense region of infected cells occupying a major portion of the nodule which is highly infested by spherical bacteroids. There are no uninfected cells interspersed among the infected ones apart from the rays of uninfected cells that divide the central infected region into two to three distinct separate masses. The outer uninfected cortical region of peanut root nodule harbours vascular bundles arranged uniformly around the central infected region.

Similar observations of peanut root nodules were discussed by Sen and Weaver (1984a) who studied the basis for different rates of nitrogen fixation by

the same strain of Bradyrhizobium in peanuts and cowpea. Comparative structural studies have shown that, unlike peanuts, the central infected region of the cowpea root nodule was interspersed by a large number of uninfected cells devoid of bacteroids. The inner side of the cortex in cowpea had an endodermis-like layer which was absent in peanut root nodules and the vascular bundles were also comparatively smaller in size in peanuts than cowpea.

The anatomical features of soybean root nodules are similar to cowpea, for the central region is heavily infected with the rhizobia and there are many small uninfected cells interspersed among the infected ones (Newcomb and Tandon, 1981a). Also, the central region is bound by an outer cortical layer which is several cells thick.

The ultrastructural studies of peanut root nodules showed a characteristic internal organization of bacteroids and host cell components which differed from the infected nodule cells of other nitrogen-fixing legumes, particularly cowpea which is also nodulated by Bradyrhizobium strain 32H1 (Sen, Weaver and Bal, 1986). The rhizobia, after infecting through peanut roots, proliferate and cause the swelling of the host cells at the point of infection. The rhizobia themselves are transformed to large spherical bacteroids with an inner and an outer membrane. The infected cells in peanut root nodules consist of these transformed bacteroids enclosed singly in the peribacteroidal membrane envelope around a central vacuole and are tightly packed. Most of the host cell components, such as mitochondria and proplastids are located near the plant cell wall. The nucleus is adpressed against the cell vacuole. In this study both lipids and microbodies have been observed in the host cytoplasm of the infected cells.

#### 4.2.1. Lipid Bodies in the Infected Root Nodule Cells.

A unique feature of peanut root nodules is the presence of lipid bodies in the infected cells during all stages of nodule development. There is a close association of the lipid bodies with the bacteroids, for they are either attached to the peribacteroidal membrane envelope or are fused with it. The squashed peanut root nodules stained with Sudan-III also give further histochemical evidence for the presence of lipid bodies. The attachment of lipid bodies to the bacteroids can even be demonstrated with the light microscope. The presence of the lipid bodies within the infected cells and their close association with the bacteroids has not been reported in the root nodules of other nitrogen-fixing legumes. The abundance of lipid bodies, their physical contact with the peribacteroidal membrane envelope and with the bacteroids suggest that the bacteroids of peanut root nodules are involved in lipid metabolism. Lipids are generally utilized through  $\beta$ -oxidation, and the catalase activity in the root nodules may be related to this pathway (Tolbert, 1981). Hydrogen peroxide is liberated during the process of  $\beta$ -oxidation which in turn should be degraded by catalase in order to neutralize its toxic effect on the cell components (Fig. 4-1).

#### 4.2.2. Microbodies.

The ultrastructural studies of peanut root nodules show the presence of microbodies in both uninfected and infected cells at all stages of nodule development. This is unlike observations in soybean where microbodies are completely absent or greatly reduced in number and size in the infected cells (Newcomb and Tandon, 1981a; Newcomb *et al.*, 1985). It was further

**Figure 4-1:** Schematic representation of fatty acid  $\beta$ -oxidation and glyoxylate pathway in germinating castor bean endosperm. Note (arrow) the formation of hydrogen peroxide through dehydrogenation of fatty acid acyl-CoA and its breakdown by the enzyme catalase. (Tolbert, 1981).



documented that the uninfected cells of soybean root nodules contain enlarged microbodies with enzymes of ureide synthesis for the production of ureides from recently fixed nitrogen. On the other hand, cowpea, like soybean, is involved in ureide metabolism, but the enzymes for ureide synthesis are reported to be present in both uninfected and infected cells (Shelp *et al.*, 1983).

Microbodies in general, have specific metabolic functions and are characterized and classified biochemically on the basis of the enzymes they possess for different metabolic pathways. They contain flavin-linked oxidases which generate hydrogen peroxide and also contain catalase for the degradation of hydrogen peroxide. The distinguishing general feature of all microbodies is the presence of catalase that can be recognized in the electron micrographs through diaminobenzidine reaction. Microbodies appear as spherical, elongate or dumb-bell shaped and have a diameter between 0.2 - 1.5  $\mu$ m. They are bound by a single, lipid bilayer membrane surrounding an amorphous or granular, electron-dense matrix, often having a dense nucleoid.

In peanut nodules the microbodies are present in close association with the bacteroids. They are seen attached to the peribacterioid membrane envelope. The occasional fusion of the peribacterioid membrane envelope and the microbody membrane is another indication of a close association between the two.

The presence of microbodies in infected and uninfected cells of peanut root nodules, and their intimate association with the bacteroids suggest that the bacteroids may be using the enzymes present in the microbodies for their own



metabolic pathways in utilizing lipids as well as for the assimilation of fixed nitrogen. It has been shown that the synthesis of allantoin, through xanthine oxidase in ureide metabolism, would generate hydrogen peroxide, which could cause damage to the cellular components (Rawsthorne, 1980). Therefore correlation of catalase activity with the effectiveness of a rhizobial strain to fix nitrogen in soybean (Francis and Alexander, 1972) is not only due to rhizobial endogenous catalase but is also due to the presence of microbodies and their association with the bacteroids.

— Continuity of microbodies with the endoplasmic reticulum often results in a pear-shaped structure which was also confirmed in this study. The density of the reaction product of DAB reaction is a qualitative measure of the catalase activity. The staining reaction of catalase/microbodies was inhibited completely or partially by KCN and by 3-amino-1,2,4-triazole (AT), suggesting that staining with DAB is related to the enzyme catalase (Fahimi, 1968, 1969). In plants the cytochemical localization of catalase in leaf microbodies through the DAB reaction was first conducted by Frederick and Newcomb (1969) through their modified technique of incubating the leaf tissues in a preincubation medium for better penetration.

2 The presence of microbodies in the infected cells of peanut root nodules along with mitochondria and lipid bodies indicates an enzymatic role of microbodies in the  $\beta$ -oxidation of fats, where the breakdown of toxic hydrogen peroxide by catalase, becomes essential. The large microbodies in the uninfected cells may be involved in ureide metabolism as has been suggested for soybean.

However, their function in peanut nodules which have a different structural organization, remains unknown. Further studies, such as the isolation of microbodies from both infected and uninfected cells, their biochemical analysis for the presence of enzymes of different metabolic pathways and their cytochemical localization within the cells, will provide more information along these lines.

#### 4.2.3. Dense Bodies.

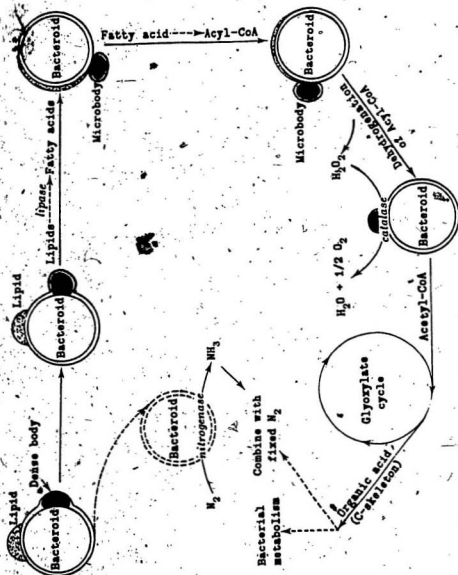
In this study of the peanut root nodule cell, unique osmiophilic structures were observed. These structures which are referred to as dense bodies resemble the inner dense region of the microbodies but are not membrane-bound. They were present at the interface between the peribacteroid membrane envelope and the outer bacteroid membrane and they were also found inside the bacteroids. The dense bodies were seen attached to the outer membrane of the bacteroids and persisted in isolated bacteroids, even after the removal of peribacteroid membrane envelope. The dense bodies had a DAB positive reaction, possibly due to catalase activity associated with the bacteroids.

In pure cultures of Bradyrhizobium, catalase activity was only found when cells were broken; there was no activity in whole bacteria. On the other hand the unbroken bacteroids from nodules, when isolated, showed very high catalase activity. It is quite possible that the catalase activity in this case was due to the dense bodies which remain attached to the outer membrane of the bacteroids. It should be mentioned that the dense bodies have not been encountered or reported in any other legume to my knowledge. Further biochemical and cytochemical work needs to be done to characterize the dense bodies and establish their

function. Because of their presence at the interface of the bacteroid and the host they are possibly playing a significant role in peanut-Bradyrhizobium symbiosis. The close association of the microbodies with the bacteroids with occasional fusion with the peribacteroidal membrane envelope and also the presence of dense bodies within the peribacteroidal membrane envelope which bulges out at the site where dense bodies are located, suggests that the membrane-bound microbodies originate from the dense bodies.

A hypothetical diagram showing the series of events involving dense bodies, microbodies and lipids is presented in Fig. 4-2. It is hypothesized that the dense bodies are surrounded by the peribacteroidal membrane envelope and are eventually pinched off into the host cytoplasm from the interface of the bacteroid and the host, as microbodies. The lipid bodies which are also in close contact with the peribacteroidal membrane envelope are broken down by the enzyme lipase into fatty acids on the surface of peribacteroidal membrane envelope (Hameed and Bal, unpublished). These fatty acids are further degraded through  $\beta$ -oxidation by a series of enzymes present in the microbodies. These microbodies are also in close contact with the peribacteroidal membrane envelope. During the process hydrogen peroxide is generated along with dehydrogenation of fatty acyl-CoA, which in turn is catabolized to water and oxygen through the catalase activity of the microbodies or the dense bodies. The lipid breakdown through  $\beta$ -oxidation results in the formation of acetyl-CoA as an end product. The process is then coupled with the glyoxylate cycle within the microbodies to produce organic acids. The organic acids may be utilized as a carbon source by the bacteroids or may combine with recently fixed nitrogen to be utilized by the host.

**Figure 4-2:** A hypothetical diagram showing the series of events related to the involvement of dense bodies, microbodies and lipids, and utilization of lipids through  $\beta$ -oxidation in peanut-Bradyrhizobium symbiosis.



## CONCLUSIONS

In the peanut-Bradyrhizobium symbiosis:

A correlation exists between the nitrogen-fixing ability and the catalase activity of peanut root nodule.

Microbodies possessing catalase activity are present in both infected and uninfected cells of the nodule. In the infected cells the microbodies are in close association with the bacteroids.

Dense bodies, which show catalase activity, are present on the surface of the bacteroids within the peribacteroidal membrane envelope.

In the in vitro grown Bradyrhizobium sp. 32H1, catalase activity is present only inside the cell, whereas in the symbiotic form within peanut root nodules, the activity is found on the surface of the bacteroid cells.

Lipid bodies are present in abundance in the nodule host cells in close association with the bacteroids. The abundance of lipid bodies, their physical contact with the peribacteroidal membrane envelope and the bacteroids, and high-catalase activity in the presence of both microbodies and dense bodies, strongly suggest that lipids are utilized as a carbon source by the peanut bacteroids during nitrogen fixation.

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